Identification and targeting of a TACE-dependent autocrine loop which predicts poor prognosis in breast cancer

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Summary

The ability to proliferate independently of signals from other cell types is a fundamental characteristic of tumor cells. Using a 3D culture model of human breast cancer progression, we have delineated a protease-dependent autocrine loop which provides an oncogenic stimulus in the absence of proto-oncogene mutation. Inhibition of this protease, TACE/ADAM17, reverts the malignant phenotype by preventing mobilization of two crucial growth factors, Amphiregulin and TGFα. We show further that the efficacy of EGFR inhibitors is overcome by physiological levels of growth factors and that successful EGFR inhibition is dependent on reducing ligand bioavailability. Using existing patient outcome data, we demonstrate a strong correlation between TACE and TGFα expression in human breast cancers that is predictive of poor prognosis**.**

Significance

Tumors resulting from inappropriate activation of the EGFR are common in multiple tissues and are, for the most part, refractory to current targeted therapies. The data presented here delineate the molecular mechanism by which constitutive EGFR activity may be achieved in tumor progression without mutation of the EGFR or downstream pathway components. These data implicate TACE as a therapeutically tractable enzyme, the inhibition of which effectively blocks EGFR signaling by preventing mobilization of ligands for this receptor. The data provide mechanistic insight into the insensitivity of EGFR-overexpressing tumors to anilinoquinazoline inhibitors and suggest that co-ordinate inhibition of TACE might augment the activity of EGFR inhibitors in a clinical setting.

Introduction

Whether achieved by gene overexpression, mutation, or amplification, the ability to grow independently of signals from other cell types is a central feature of tumorigenesis, and the acquisition of self-sufficiency for growth signals is a critical rate-limiting transition in the evolution of a tumor cell (Hanahan and Weinberg, 2000). Pathways downstream of the Epidermal Growth Factor Receptor (EGFR) play essential roles in cell proliferation. Genetic ablation of this receptor or of certain ligands impairs

mammary gland development (Luetteke et al., 1999; Wiesen et al., 1999), while deregulated ErbB pathway signaling contributes to a significant proportion of human cancer cases, both in the breast and in other tissues (Downward, 2003). Thus, appropriate spatial and temporal regulation of EGFR signaling is crucial for correct mammary gland development and for the maintenance of mammary epithelial organization.

We have used three-dimensional (3D) culture models of breast epithelial cells to investigate the molecular determinants of constitutive growth factor receptor signaling and to evaluate the phenotypic consequences of targeting these processes. The HMT3522 breast cancer progression series originated from purified human breast epithelial cells derived from reduction mammoplasty (Briand et al., 1987). Early passages (S1) are spontaneously immortalized, non-malignant cells which require exogenous EGF for proliferation (Briand et al., 1987) and retain the capacity to differentiate into growth-arrested, polarized acinar structures with central lumina when cultured in 3D gels of extracellular matrix (Petersen et al., 1992). Later passages (T4-2) grow independently of exogenous EGF and are tumorigenic *in vivo* (Briand et al., 1996). They fail to arrest growth in the 3D assay and form large, continuously proliferating apolar colonies. As these cells are ERα-negative and EGFR/ERBB2-positive, they are representative of a class of breast tumors with poor prognosis (Slamon et al., 1987; Sommer and Fuqua, 2001). Treatment of T4-2 cells in 3D culture with inhibitors of components of the EGFR pathway elicits a striking morphological reversion of this malignant behavior and the assumption of an organized, growth-arrested, polarized acinar structure (Wang et al., 1998).

Here, we use this model to investigate the mechanisms by which non-malignant breast epithelial cells may escape dependence on exogenous EGF. A dissection of the EGFR pathway in T4-2 cells revealed that these cells lack mutations in common proto-oncogenes (H-Ras, K-Ras, N-Ras and B-Raf) but express two EGFR ligands not present in S1 cells, Amphiregulin (AREG) and TGFα. A metalloproteinase activity, TACE/ADAM17, implicated by others in processing of these ligands (Borrell-Pages et al., 2003; Gschwind et al., 2003; Sahin et al., 2004), is expressed in T4-2 cells and is necessary for AREG and TGFα function. We show that inhibition of TACE is sufficient to block EGFR signaling and to revert the malignant phenotype in T4-2 cells and that this

is a direct consequence of attenuation of growth factor ectodomain shedding. Analysis of a published microarray dataset encompassing 295 primary breast tumors and associated clinical data (van de Vijver et al., 2002) revealed that TACE and TGFα expression are highly correlated and predictive of a poor prognosis.

These data provide further insight into this fundamental feature of tumorigenesis in the breast and other epithelial tissues and suggest that targeting this ADAM-dependent autocrine loop may prove an entirely new therapeutic strategy for EGFR-dependent tumors. In this regard, we have recognized the lack of success of EGFR inhibitors in recent phase III clinical trials (Giaccone et al., 2004; Herbst et al., 2004) and have hypothesized that availability of ligands may counter the efficacy of these drugs. We show that the reversion of malignancy induced by EGFR inhibition is overcome by low picomolar quantities of EGFR ligands – concentrations within the normal physiological range of these proteins. These data suggest that approaches which limit EGFR ligand bioavailability (such as TACE inhibition) might augment the effect of EGFR inhibitors in a clinical setting.

Results

Amphiregulin and TGFα are upregulated in T4-2 cells

S1 non-malignant human breast epithelial cells require exogenous EGF for proliferation (Figure 1B,C), while their malignant derivatives, T4-2, have acquired self-sufficiency for this signal. The sensitivity of T4-2 cells to inhibition of EGFR (Wang et al., 1998) implies that EGFR and the downstream components of the pathway are not mutationally activated. Using direct sequencing, we showed that these cells have not sustained activating mutations in H-Ras, K-Ras, N-Ras or B-Raf (data not shown). Thus, we hypothesized that T4-2 cells escaped dependence on exogenous EGF by transcriptionally upregulating one or more ErbB ligands. We tested expression of Amphiregulin, Betacellulin, Cripto, EGF, Epiregulin, HB-EGF, NRG1, NRG2 and TGFα by RT-PCR. Amphiregulin and TGFα were expressed at high levels in T4-2 cells (Figure 1A). Experiments using concentrations of recombinant AREG or TGFα equimolar to that of EGF (860 pM) show that these ligands can substitute for EGF to promote proliferation of the non-malignant cells (Figure 1D-F).

By the time an incipient cancer cell has become malignant, it is characterized by multiple genomic mutations, chromosomal amplifications and deletions, and aneuploidy (Albertson et al., 2003; Rajagopalan et al., 2003). In such a chaotic background, it is often difficult to distinguish between causative changes, correlative changes and changes of little consequence. If the changes we have observed in expression of these genes in malignant T4-2 cells are indeed important, one might expect that some of them would be detected at earlier stages of progression. On the continuum between S1 and T4-2 cells, a subline was established which, like S1 cells, is non-malignant but which grows independently of EGF and has lost the ability to form growth-arrested polarized acinar structures in 3D lrECM culture. This subline, S2 cells, was derived from S1 cells by EGF withdrawal after 118 passages (Briand et al., 1996). Analysis of the expression of AREG and TGFα in S2 cells (Figure 1G) demonstrates that both of these factors were upregulated during the transition from S1 to S2, the earliest stage of progression toward malignancy in this series.

A metalloproteinase activity is critically required for mobilization of growth factors

Several growth factors, including AREG and TGFα, are synthesized as transmembrane precursors and members of the ADAM family of transmembrane proteases have been implicated in the processing of these ligands (Borrell-Pages et al., 2003; Gschwind et al., 2003; Sahin et al., 2004). Culture of T4-2 cells in 3D extracellular matrix results in the formation of disorganized, apolar, continuously proliferating colonies (Figure 2A), a phenotype we have shown to be highly correlated with, and reflective of, cancer cell malignancy (Petersen et al., 1992; Wang et al., 2002). Incubation with TAPI-2, a broad-spectrum inhibitor of MMPs and ADAMs, resulted in a reversion of the malignant phenotype (Figure 2C) similar to that elicited using the EGFR inhibitor AG1478 (Figure 2B), suggesting that a metalloproteinase activity is required for the proliferative phenotype of T4-2 cells. This treatment also resulted in the restoration of epithelial polarity. Vehicle-treated cells remained disorganized (Figure 2D) while TAPI-2 treated cells assumed a polar organization similar to that of a breast acinus, here indicated by basal localization of α6-integrin (Figure 2E). The colonies formed by AG1478 or TAPI-2 treated T4-2 cells were similar in size to non-malignant mammary acini and were significantly smaller than those formed by cells treated with vehicle alone (Figure 2F).

T4-2 cells exhibit a basal level of activity of signaling kinases downstream of the EGFR (Figure 2G, lane 1) which is consistent with a response to the ongoing production of an EGFR ligand by these cells. The basal activities were significantly suppressed by addition of TAPI-2 (Figure 2G, lane 3) but the cells remained competent to respond to addition of exogenous EGF (Figure 2G, lane 4). Furthermore, TAPI-2 caused a dose-dependent decrease in proliferation of T4-2 cells in 2D cultures, which was overcome by addition of exogenous EGF (Figure 2H). This compound was not cytotoxic at the concentration used, nor did it interfere with the ability of S1 cells to execute normal acinar morphogenesis in the presence of soluble EGF (data not shown). Thus, the proliferative block and concomitant reversion resulting from metalloproteinase inhibition appears to result, at least in part, from a defect in growth factor mobilization, suggesting that either an MMP or ADAM family member plays a crucial role in the regulation of EGFR signaling in this transition to growth factor autonomy.

TACE/ADAM17 cleaves both AREG and TGFα in cultured mammary epithelial cells

Several lines of genetic and biochemical evidence suggest that TACE/ADAM17 is a key regulator of cleavage of both AREG and TGFα (Borrell-Pages et al., 2003; Gschwind et al., 2003; Sahin et al., 2004). TACE is expressed in both S1 and T4-2 cells (Figure 3A). To test whether TACE could cleave endogenously produced growth factors in mammary epithelial cells, we cloned and overexpressed the transmembrane precursors of Amphiregulin and TGFα in S1 cells. Acute stimulation of these cells with recombinant TACE was sufficient to mobilize the growth factors to activate receptor tyrosine kinase signaling (Figure 3B), a response not elicited in the vector control cells. Introduction of siRNAs against TACE significantly suppressed T4-2 cell proliferation compared to both GFP-transfected and random siRNA-transfected controls (Figure 3C). Thus it appears that TACE, and not another TAPI-2-sensitive protease, is the primary growth factor sheddase in T4-2 cells.

AREG and TGFα are the key substrates of TACE in T4-2 cells

In addition to shedding growth factors, TACE has been implicated in the shedding of several cell surface molecules, inhibition of which might also contribute to the observed reversion of the T4-2 cell phenotype. Characterized substrates of TACE include

TNFα (Black et al., 1997; Moss et al., 1997), L-Selectin and TNFRII (Peschon et al., 1998), β-APP (Buxbaum et al., 1998), collagen XVII (Franzke et al., 2002), growth hormone receptor (Zhang et al., 2000), TrkA (Diaz-Rodriguez et al., 2002), ErbB4 (Rio et al., 2000) and GPIbα (Bergmeier et al., 2004). To test whether modulation of growth factor cleavage is the key role of TACE here, we generated soluble secreted mutants of both AREG and TGFα which lack both the transmembrane and cytosolic domains (Figure 4A). and sought to determine whether their overexpression might lead to a genetic rescue of the TAPI-2-imposed reversion.

Each stably transfected T4-2 cell line was susceptible to reversion by EGFR inhibition (Figure 4B). Those cells which produced soluble growth factors were completely resistant to TAPI-2 (Figure 4B). Thus, despite the number of TACE substrates expressed by these cells, it is the suppression of growth factor mobilization which results in the reversion of the malignant phenotype.

EGFR ligand bioavailability antagonizes inhibitor efficacy

Small molecule inhibitors of the EGFR have thus far proven disappointing in phase III clinical trials. Like AG1478, Gefitinib (Iressa, ZD1839) and Erlotinib (Tarceva, OSI-774) are reversible anilinoquinazoline-derivatives. In two large trials of non-small cell lung cancer (NSCLC) patients, Chemotherapy with Gefitinib performed no better than chemotherapy alone in terms of survival (Giaccone et al., 2004; Herbst et al., 2004). Although Erlotinib did provide a statistically significant survival benefit in patients with advanced pancreatic adenocarcinoma, it is important to note that the median extension in progression-free survival was a mere six days, while the median increase in overall survival was 14 days (Moore, M.J. et al. Erlotinib improves survival when added to gemcitabine in patients with advanced pancreatic cancer. A phase III trial of the National Cancer Institute of Canada Clinical Trials Group [NCIC-CTG]. *ASCO Gastrointestinal Cancers Symposium* (2005) Abstract 77). In a recent trial using Erlotinib as a single agent in NSCLC, median progression-free survival time was increased by almost two weeks in the treated population, while median overall survival was increased by two months (Shepherd et al., 2005).

Despite the fact that T4-2 cells expressing soluble AREG or TGFα still formed small rounded colonies in the presence of the EGFR inhibitor, these colonies were

consistently larger than those formed by cells infected with either empty vector or with the membrane-tethered pro-forms of AREG and TGFα (Figure 4B). The difference was statistically significant (median cross-sectional area of AG1478-treated proAREG v AREGΔTM colonies, $P < 0.0001$; proTGFα v TGFαΔTM, $P < 0.05$). We postulated that successful inhibition of the EGFR by anilinoquinazoline inhibitors might be a function of the availability of circulating ligand and tested this hypothesis in a quantitative fashion in T4-2 cells (Figure 4C). The malignant phenotype of T4-2 cells (typically cultured in the absence of EGF) may be reverted using AG1478. Increasing the amount of soluble EGF allowed the T4-2 cells to escape from the AG1478-imposed reversion (Figure 4C). Similar observations were made with both recombinant Amphiregulin and TGFα (data not shown). Thus it seems that there is a transition point of ligand concentration above which EGFR inhibitors lack efficacy. In the 3D culture assay, this transition occurred between 0.1 and 1 ng/ml. Reported levels of EGF and TGFα (both circulating and in tumor and tissue homogenates) are within or exceed this range (Messa et al., 1998; Reeka et al., 1998; Sotnikova et al., 2001).

TACE and TGFα predict poor prognosis in human breast cancer patients

Having thus established that TACE-dependent growth factor shedding plays a role that is both critically important and therapeutically tractable in this model of breast cancer progression, we sought to determine the extent to which these factors play a role in human breast cancer. We interrogated a comprehensive microarray dataset of 295 primary human breast tumors which was prepared by Marc van de Vijver and colleagues, who used it to identify gene expression signatures predictive of outcome. The detailed clinical characteristics of these tumors have been reported (van de Vijver et al., 2002). Briefly, all were either stage I or II and less than 5 cm diameter at excision, and derived from 295 consecutively treated patients less than 53 years old. Approximately three quarters of the tumors were ERα positive, and half were associated with positive lymph nodes. The median time for which follow-up information is available is 6.7 years (range 0.05 – 18.3 years).

Our analysis of this publicly available dataset revealed a statistically significant positive correlation between expression levels of TGFα and TACE (Table 1, $P < 0.001$). EGFR expression also tended to correlate with both TGFα and TACE, although not quite

reaching the level of statistical significance ($P = 0.053$ and $P = 0.061$, respectively). Interestingly, Amphiregulin expression in this patient population was inversely correlated with expression of EGFR and TGFα ($P < 0.05$ and $P < 0.001$, respectively). Amphiregulin and TACE levels tended to be anti-correlated, although not quite reaching statistical significance ($P = 0.053$). While these three markers were co-expressed in our progression model, these data suggest that TACE and TGFα may be the more important protease/growth factor pair for EGFR activation in human breast tumors. Tumors positive for TGFα, ADAM17 and EGFR tended to be ERα negative ($P < 0.0001$, $P < 0.005$, $P < 0.0001$ respectively). Conversely, ERα positive tumors tended to have higher levels of Amphiregulin ($P < 0.0001$).

To analyze the contribution of AREG, TGFα, and TACE expression to survival, tumors were divided in quartiles by expression level of each marker and survival curves were computed for the upper and lower quartiles (74 samples each) and the interquartile range (147 samples). High levels of TACE expression were associated with a poor survival (Figure 5A, $P < 0.05$, high v. low expression). Tumors with the highest levels of TGFα expression also tended to have a poorer outcome, although statistically this was borderline (Figure 5B, $P < 0.06$). Tumors which express high levels of Amphiregulin had a significantly better outcome than tumors expressing lower levels (Figure 5C, $P < 0.001$) as expected from the high correlation between Amphiregulin and ERα expression levels, positivity for the latter being a strong predictor of survival (Figure 5D, $P < 0.001$).

These data illuminate the necessary steps, at a molecular level, by which tumor cells may become independent of extrinsic proliferative signals and suggest that ADAM family members may prove important additional therapeutic targets in EGFR-dependent malignancies of the breast and other tissues.

Discussion

In this study, we show that activation of EGFR signaling in the T4-2 cells of the HMT3522 breast cancer progression series is driven by a TACE-dependent growth factor autocrine loop not present in the non-malignant S1 cells. Inhibition of TACE attenuated the growth of T4-2 cells in 3D ECM culture and reverted their morphology to approximate that of non-malignant cells. This reversion was overcome by overexpression of soluble "pre-cleaved" mutants of either AREG or TGFα but not by pro-AREG or pro-

TGFα, definitively demonstrating the importance of growth factor precursor cleavage for ErbB function. We further show that expression of TACE and TGFα is highly correlated in human breast cancer samples and is predictive of poor prognosis. Lastly, we demonstrate that ligand bioavailability, which can be modulated by TACE inhibition, is an important determinant of EGFR inhibitor efficacy.

Despite the development of potent, specific EGFR inhibitors, EGFR-dependent tumors of several tissues remain a substantial clinical problem. Some patients who do respond to therapy have tumors bearing EGFR mutations (Lynch et al., 2004; Paez et al., 2004), but this explained only a proportion of responses in these studies, and the association has not been reproduced in another large study (Tsao et al., 2005). Our data demonstrate that the utility of an EGFR inhibitor may be a function of the abundance of ligand and that low picomolar levels of these growth factors can overcome the efficacy of the inhibitor. If so, physiological levels of EGFR ligands may antagonize the action of EGFR inhibitors *in vivo*. Among the small proportion of patients whose tumors respond to small molecule EGFR inhibitors, there is frequent and pronounced adverse systemic reactions to the drug, including skin rash and diarrhea. Intriguingly, these systemic responses predict response to Gefitinib (Chiu et al., 2005; Mohamed et al., 2005; Perez-Soler et al., 2004). The fact that systemic toxicity predicts response is consistent with the hypothesis that circulating and locally produced EGFR ligands antagonize Gefitinib efficacy in both the tumor and in other organs: Those patients with circulating ligands above a threshold would experience neither skin rashes nor tumor regression in response to Gefitinib. This hypothesis is supported by our demonstration in 3D cultures that EGFR inhibitor efficacy is critically dependent on the bioavailability of EGFR ligands, and that ligand concentrations within the normal circulating range (Messa et al., 1998; Reeka et al., 1998; Sotnikova et al., 2001) confer resistance to the inhibitor. Interestingly, Amphiregulin overexpression predicts non-responsiveness to Gefitinib in ERRB2-positive non-small cell lung cancer tumors (Kakiuchi et al., 2004). Undoubtedly in some cases Gefitinib resistance is acquired in a cell-autonomous fashion by tumor cells, by loss of PTEN (She et al., 2003), by acquisition of mutation in EGFR (Pao et al., 2005), or perhaps by switching dependence to other ErbB family members. However, it is difficult to understand how such changes within the primary tumor could ameliorate the systemic

toxicity. Thus we believe that systemic resistance to EGFR inhibitors, resulting from an excess of circulating EGFR ligands, may be an important predictive determinant for anilinoquinazoline efficacy and that reduction of the levels of circulating growth factors by inhibiting growth factor shedding may improve the efficacy of these compounds.

The demonstration of an absolute requirement for an ADAM-like proteolytic activity for proliferation in a physiologically relevant model of human breast cancer progression suggests another avenue to be explored therapeutically. MMPs and ADAMs have been studied intensively and many small molecule inhibitors have been characterized in both cell culture and animal models, primarily to inhibit MMP-dependent tumor cell invasion. Despite the relative success of pre-clinical studies, the results of many clinical trials of MMP inhibitors in cancer have been disappointing, perhaps due to what retrospectively appears to be flawed design of the Phase III studies (Coussens et al., 2002). It has become clear that metalloproteinases play more complex and diverse roles in tumor progression (Egeblad and Werb, 2002) than was appreciated during the design of these earlier clinical studies. It remains possible that these compounds may prove efficacious in selected subsets of patients, one such cohort being those who depend on TACE-dependent autocrine stimulation of EGFR/ErbB2. Specific and orally active TACE inhibitors have been developed by Roche (Beck et al., 2002) and Wyeth (Zhang et al., 2004) for treatment of arthritis; our data suggest that their efficacy should be evaluated in a pre-clinical models of EGFR ligand-dependent tumorigenesis.

Our data delineate a mechanism by which breast epithelial cells may escape dependence on extrinsic proliferative signals, a transition necessary in the evolution of all cancers. The essential role of TACE in this phenotype, and the demonstration that inhibition of this protease blocks EGFR signaling and reverts the malignant phenotype suggests that interruption of such an autocrine loop might prove an effective therapy for tumors dependent on EGFR ligand expression, alone or in combination with existing EGFR inhibitors.

Experimental Procedures

Cell culture All reagents were purchased from SIGMA (St. Louis, MO) except where otherwise noted. HMT3522 cells were cultivated on 2D and 3D substrata in H14 medium, a 50:50 mix of DMEM/F12 (UCSF Cell culture Facility, San Francisco, CA)

supplemented with 5 μg/ml prolactin, 250 ng/ml insulin, 1.4 x 10^{-6} M hydrocortisone, 10^{-10} M β-estradiol, 2.6 ng/ml sodium selenite and 10 μg/ml transferrin. S1 cells were additionally supplemented with 10 ng/ml EGF. In various experiments, cells were supplemented with AREG, TACE, TGFα. In all cases, AREG and TGFα were used at the same molar concentration as EGF (860 pM).

For 3D lrECM culture, T4-2 cells were seeded at 21000 cells per cm^2 on top of Matrigel, overlaid with H14 medium containing 5% Matrigel (BD Biosciences, San Jose, CA), and treated with 80 nM AG1478, 20 μM TAPI-2 or the relevant vehicle controls.

Amphotropic retroviruses were generated by transfection (Lipofectamine; Invitrogen, Carlsbad, CA) of the Phoenix packaging cell line (a gift of Dr. Gary Nolan, Stanford) with pBM-IRES-Puro or derivatives containing the AREG or TGFα open reading frames. Two million phoenix cells per 6 cm dish were plated the day prior to transfection and transfected with 2 μg of the appropriate retroviral construct. Retrovirus-containing culture medium was harvested after 48 hrs, supplemented with polybrene to 5μg/ml and added to HMT3522 cells at 30-50% confluence. Pools of stable infectants were selected in 1 μg/ml puromycin.

Silencer™ siRNAs against TACE (Ambion, Austin, TX) were co-transfected with pEGFP-C1 (BD Biosciences). T4-2 cells were trypsinized post-transfection and plated at low density. Proliferation was assessed by counting the transfected (green) cells per colony after four days. Random siRNA sequence was used as a negative control.

Indirect immunofluorescence. Colonies were solubilized from matrigel culture by shaking in PBS/0.05M EDTA on ice for 30 mins, fixed in 4% paraformaldehyde, permeabilized, stained with anti-α6-integrin (Chemicon, Temecula, CA) and counterstained with DAPI.

Western blotting. Cells were lysed in 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.5% NP40 supplemented with protease and phosphatase inhibitors (Calbiochem, San Diego, CA) and clarified by centrifugation. 50 μg of each sample was fractionated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies against the following proteins: phospho-MAPK, Total MAPK, phospho-p70S6-Kinase, (Cell Signaling Technology, MA). E-cadherin (BD Biosciences, CA) was used as a loading

control. Blots were developed using Supersignal West Femto (Pierce, Rockland, IL). Images were captured using a FluorChem 8900 imager (Alpha Innotech, San Leandro, CA).

Cloning of pro-AREG and pro-TGFα. The open reading frames of these genes were amplified by PCR from T4-2 cDNA. Amplification products were cloned, sequence verified, and subcloned into the retroviral expression vector, pBM-IRES-Puro (Garton et al., 2002). The primers used were: AREG: 5'-GACCTCAATGACACCTACTCTGG-3', 5'-GAAATATTCTTGCTGACATTTGC-3' ; TGFα: 5'-ATGGTCCCCTCGGCTGGACAGCTC-3', 5'-TCATAGATCTTCTTCTGATATAAGCTTTTGTTCGACCACTGTTTCTGAGTGGC-3'. The ΔTM mutants of AREG and TGFα were generated from the using the pBM-IRES-puro specific primer 5'-TGGAAAGGACCTTACACAGTCC-3' and either 5'-AAAAGGATCCTCATTTTGATAAACTACTGTCAATC-3' (AREGΔTM) or 5'-AAAAGGATCCTCAGGCCTGCTTCTTCTGGCTGGC-3' (TGFαΔTM) and cloned into pBM-IRES-Puro.

Proliferation assays. HMT3522 cells were seeded in 96 well plates and treated (in triplicate) as described in the figure legends. To determine relative growth, 0.1 volumes of WST cell proliferation analysis reagent (Roche, Indianapolis, IN) was added to the medium and its formazan metabolite was measured by absorbance at 460 nm.

RTPCR. DNase-treated total RNA was isolated using the RNEasy kit (Qiagen, Valencia, CA). 5 μg of total RNA in a final volume of 40 μl was used for oligo dT primed cDNA synthesis (First Strand cDNA synthesis kit, Invitrogen, Carlsbad, CA). 1 μl of cDNA was added to a 60 μl PCR reaction. 15 μl aliquots were withdrawn after 25, 30 and 35 cycles and analyzed by agarose gel electrophoresis. Primers used were as follows: AREG: 5'-GACCTCAATGACACCTACTCTGG-3', 5'-GAAATATTCTTGCTGACATTTGC-3' ; GAPDH: 5'-CCCCTGGCCAAGGTCATCCATGAC-3', 5'-CATACCAGGAAATGAGCTTGACAAAG-3' ; TACE: 5'-CAGCACAGCTGCCAAGTCATT-3', 5'-CCAGCATCTGCTAAGTCACTTCC-3'; TGFα: 5'-CACACTCAGTTCTGCTTCCA-3', 4'-TCAGACCACTGTTTCTGAGTGGC-3'.

Statistical Analyses. All data analysis was performed using Graphpad Prism. Bar graphs represent mean ± standard error of mean. Significance was determined using ANOVA. In scatter plots, the horizontal bar represents the median of each dataset. Significance was determined using Kruskal-Wallis test (with Dunn's Test to correct for multiple comparisons).

A database consisting of the microarray profiles of 295 human breast tumors with the associated clinical data (van de Vijver et al., 2002) was obtained from Rosetta Inpharmatics. Pearson's correlation coefficient was used to determine whether statistically significant associations existed between the relative expression levels of each of the markers. For survival analysis, patients were stratified into quartiles for expression of each marker, and survival curves computed using the method of Kaplan and Meier. Statistical significance was determined using the log-rank test.

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Table 1 Pearson's correlation analysis of markers in 295 primary human breast tumors

	AREG		ERα		TGFα		ADAM17		EGFR	
	r	p	r	p	r	p	r	p	R	p
ERα	0.4177	<0.0001								
TGFα	-0.2155	0.0002	-0.3851	<0.0001						
ADAM17	-0.1126	0.0534	-0.1774	0.0022	0.1917	0.0009				
EGFR	-0.1416	0.0150	-0.2755	<0.0001	0.1127	0.0532	0.1093	0.0609		
ERBB2	0.1897	0.0011	-0.0943	0.1062	-0.1027	0.0783	-0.0340	0.5609	-0.0557	0.3407

FIGURE LEGENDS

Figure 1. Amphiregulin and TGFα are upregulated in T4-2 cells and can substitute for EGF to promote proliferation of S1 cells

A: RT-PCR analysis shows that both Amphiregulin and TGFα are transcriptionally upregulated in T4-2 relative to S1 cells.

B: S1 cells cultured for six days in the absence of EGF do not proliferate.

C-E: S1 cells proliferate in the presence of equimolar (860 pM) of each EGFR ligand.

F: S1 cell proliferation in the presence of each ligand is significantly different from control.

G: RT-PCR analysis shows that both Amphiregulin and TGFα are upregulated in non-malignant S2 cells.

Figure 2. Inhibition of sheddase activity reverts the malignant phenotype of T4-2 cells by suppressing mobilization of growth factors and downregulating EGFR pathway activity

A: T4-2 cells grown in 3D lrECM culture form continuously proliferating, disorganized, and apolar colonies.

B: T4-2 cells treated with EGFR inhibitor (80 nM AG1478) undergo morphological reversion, forming small, smooth, spherical, growth-arrested colonies.

C: T4-2 cells treated with a broad-spectrum MMP/ADAM inhibitor (20 μM TAPI-2) undergo a morphological reversion similar to that of EGFR inhibitor-treated cells. Bar = 100 μm.

D: α6-integrin staining of T4-2 cells indicates absence of tissue polarity.

E: α6-integrin staining of T4-2 cells shows restoration of tissue polarity in TAPI-2 treated cells. Bar = 10 μm.

F: Analysis of cross-sectional area of T4-2 cells treated with vehicle, AG1478 or TAPI-2 for four days. Both AG1478 and TAPI-2 treated colonies were significantly different from controls ($p < 0.001$).

G: TAPI-2 treatment (24 hours) reduces the basal activity of kinases downstream of EGFR, but cells remain competent to respond to exogenous EGF (860 pM, 5 minute stimulation).

H: TAPI-2 treatment results in a dose-dependent reduction in T4-2 cell proliferation that is completely overcome by addition of soluble EGF.

Figure 3. TACE/ADAM17 cleaves both Amphiregulin and TGFα, and promotes T4-2 cell proliferation

A: RTPCR analysis showing TACE expression in both S1 and T4-2 cells.

B: Stimulation with recombinant TACE (5 mins) activated EGFR signaling in S1 cells overexpressing full-length Amphiregulin or TGFα, but not in vector-transfected cells.

C: Suppression of TACE expression reduced T4-2 cell proliferation.

Figure 4. TAPI-2 induced reversion of T4-2 cells is a direct result of inhibition of growth factor ectodomain shedding

A: Schematic representation of full-length and deletion mutants of Amphiregulin and TGFα. ΔTM mutants lack both the transmembrane and cytoplasmic domain and are thus secreted without requiring TACE activity.

B: T4-2 cells overexpressing both full-length and deletion growth factor constructs are susceptible to reversion induced by the EGFR inhibitor, but those cells expressing either soluble Amphiregulin or TGFα escape the TAPI-2-imposed reversion. Bar = 100 μm.

C: Reversion of T4-2 cells by EGFR inhibition is overcome by the presence of picomolar amounts of EGFR ligand. Cells were cultured for four days in 80 nM AG1478 and various amounts of EGF. Above a threshold EGF concentration between 0.1 - 1 ng/ml (8.6 - 86 pM), the EGFR inhibitor can no longer revert the malignant phenotype. Bar = 100 μm.

Figure 5. Kaplan-Meier survival analysis of 295 human breast tumors stratified by marker expression level

High levels of (**A**) TACE and (**B**) TGFα predict poor survival. High levels of (**C**) Amphiregulin or (**D**) ERα are predictive of survival. *P* values represent the log-rank comparison between the upper and lower quartiles of marker expression.

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Figure 1

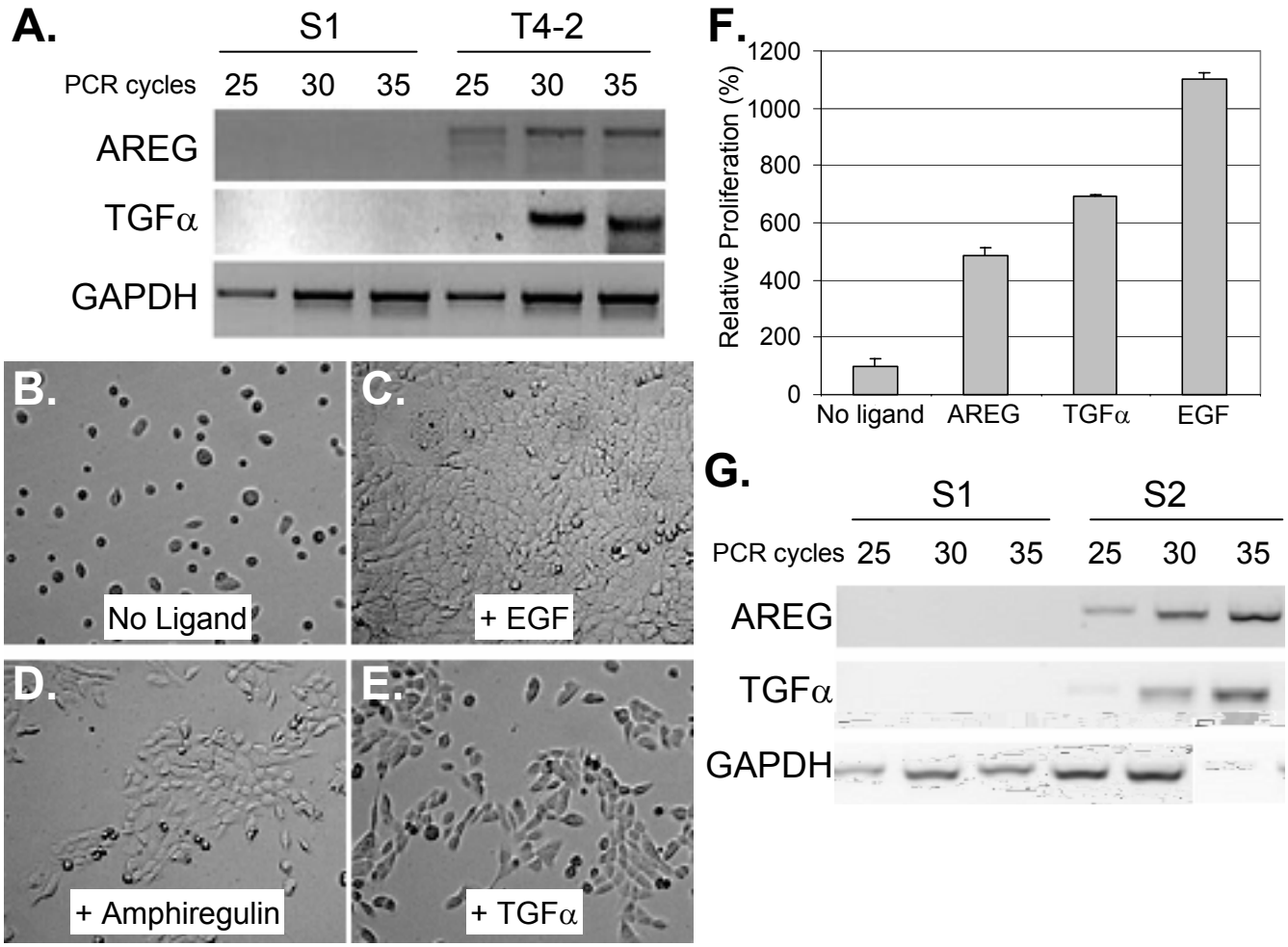

Fig 1

Figure 2

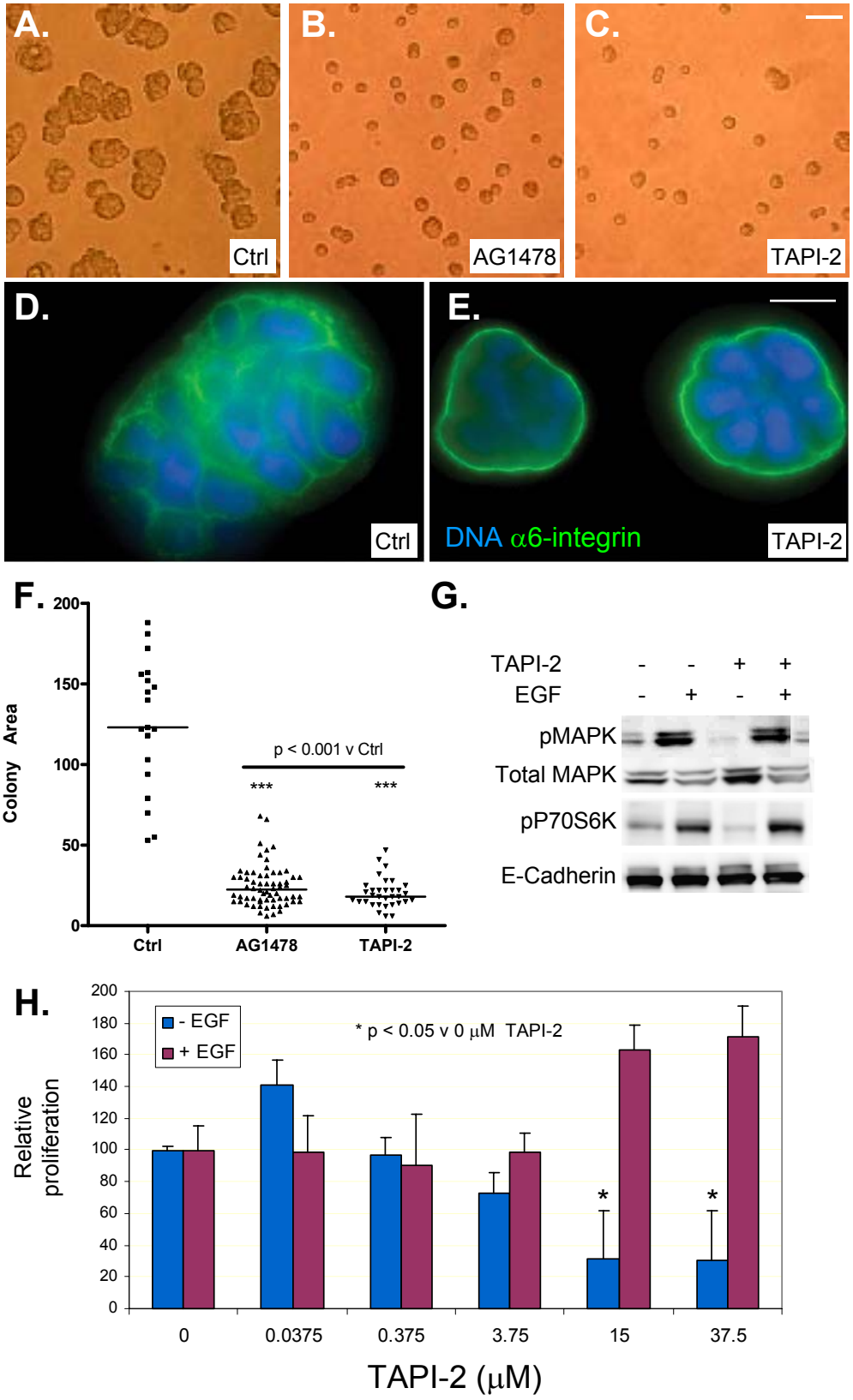

Fig 2

Figure 3

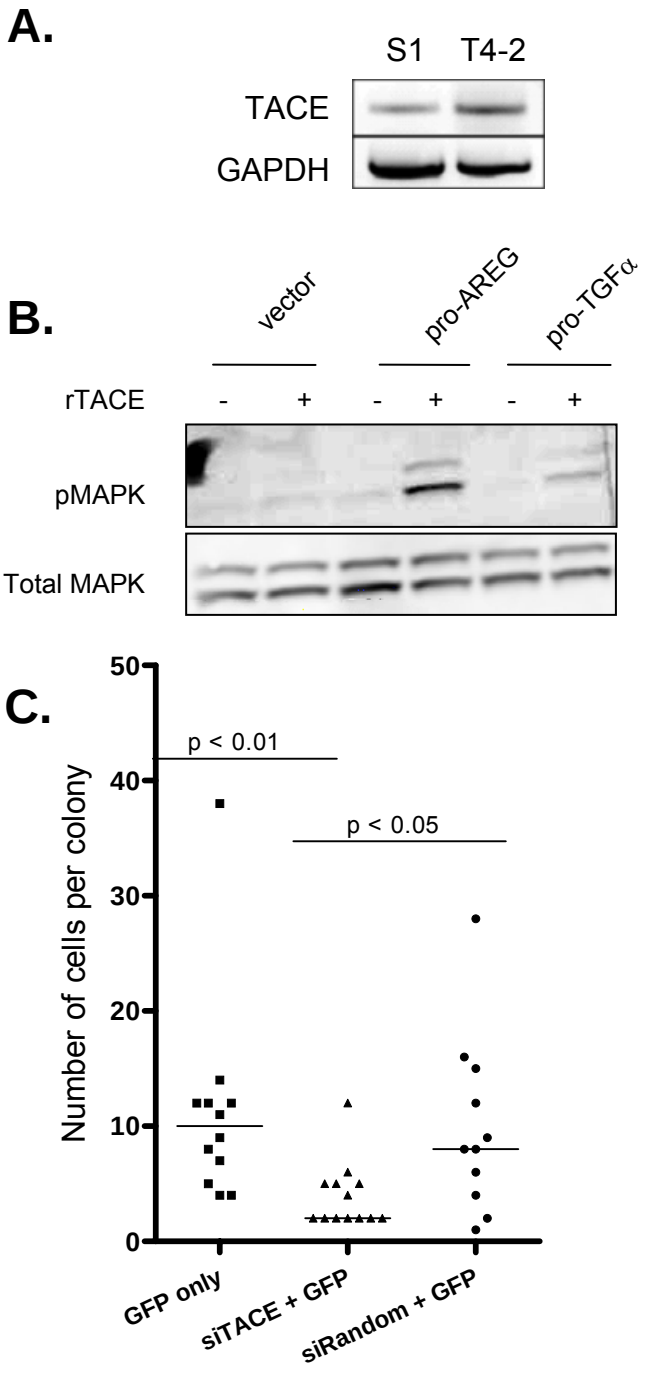

Fig 3

Figure 4

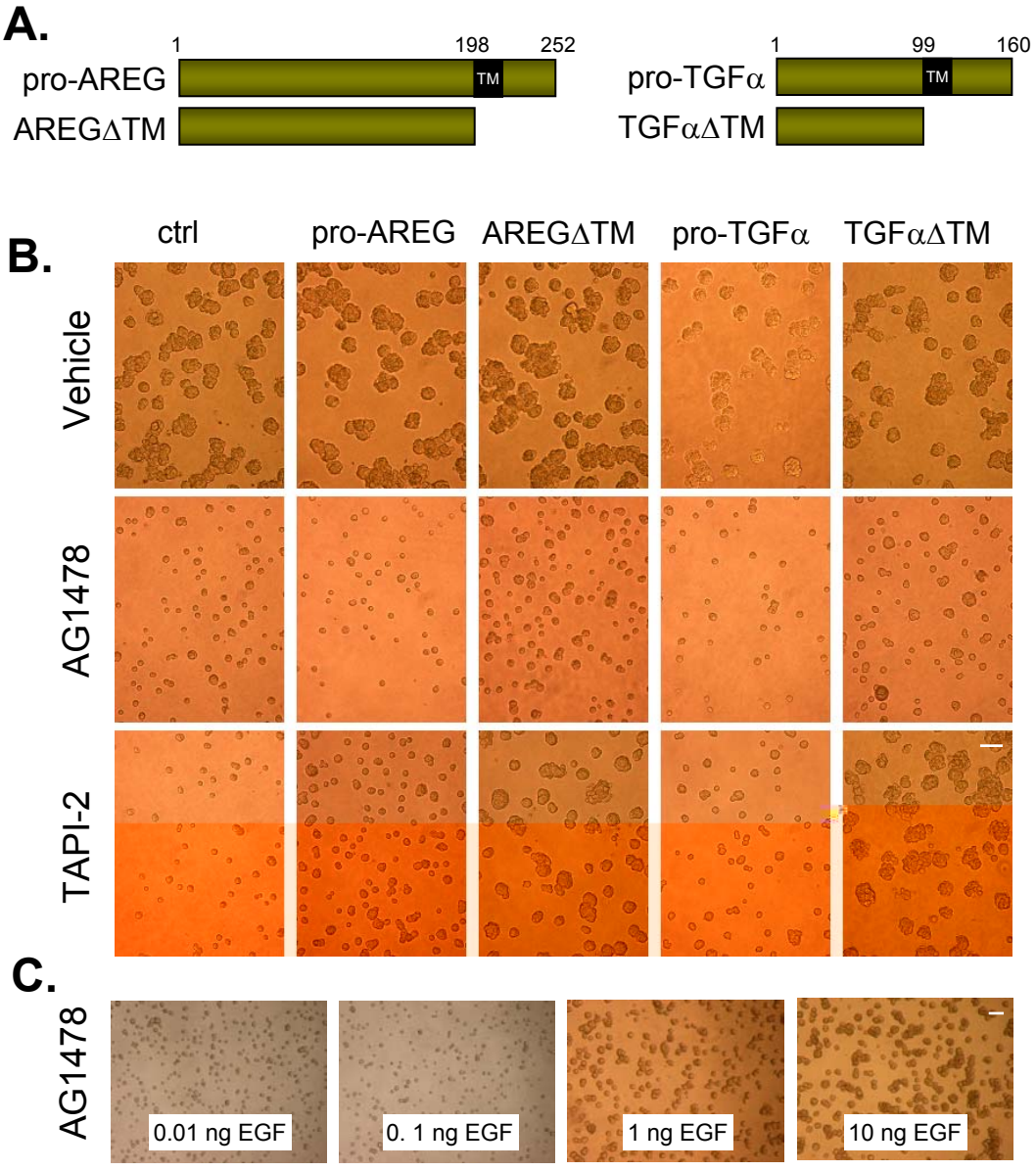

Fig 4

Figure 5

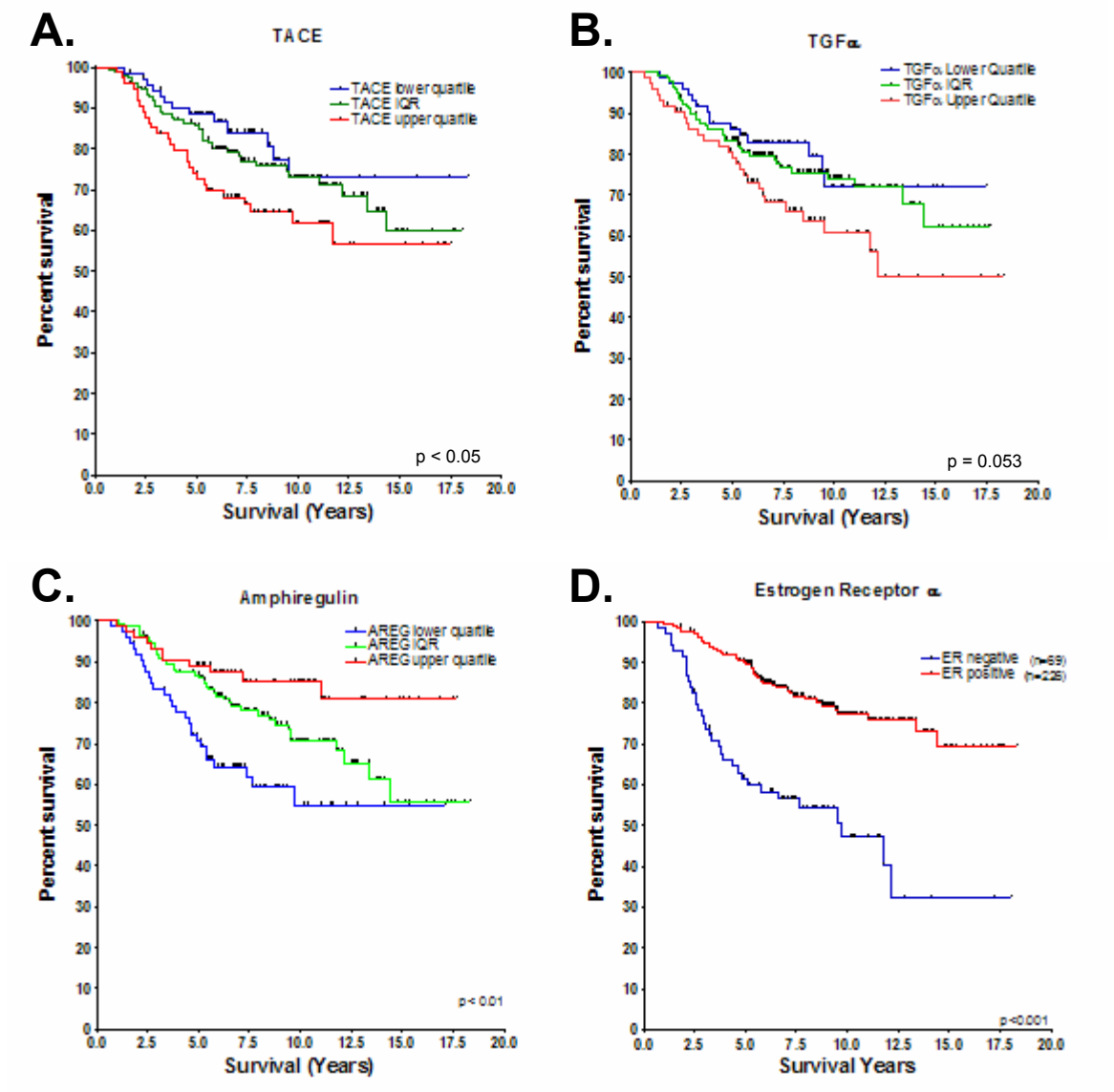

Fig 5